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(54) Title: GENE TRANSFER IN POULTRY BY INTRODUCTION OF EMBRYO CELLS *IN OVO*

(57) Abstract

A method of altering the phenotype of a bird comprises introducing a DNA sequence into somatic cells of a bird contained within an egg during *in ovo* incubation. The DNA sequence is selected to be effective to cause a change in phenotype, such as an increase in growth rate, feed efficiency, or both in the bird after hatch. A DNA sequence may further be selected to increase disease resistance or induce disease prevention by the expression of an antigen over a period of time.

GENE TRANSFER IN POULTRY BY INTRODUCTION OF EMBRYO CELLS IN OVO

Related Applications

This application is a continuation-in-part of U.S. Patent Application Serial No. 07/826,030, filed 27 January 1992, the content of which is herein incorporated by reference in its entirety.

Field of the Invention

This invention relates to the methods of altering the phenotype of birds by introducing avian embryo cells into an egg containing the bird prior to hatch, which embryo cells carry heterogenous genetic material.

Background of the Invention

Commercial poultry is an extremely important source of food. However, there has been comparatively little attention given to methods of producing useful changes in the phenotype of birds through genetic engineering techniques. This is unfortunate, because such techniques offer a much more rapid technique for introducing desirable phenotypic traits into birds than classical breeding techniques.

In view of the foregoing, an object of the present invention is to provide methods of changing the phenotype of birds through genetic engineering procedures.

An additional object of the present invention is to provide a method of changing the phenotype of birds in which expression of an exogenous DNA sequence is sufficient produce the phenotypic change.

Another object of the present invention is to provide a method of changing the phenotype of birds which is rapid and convenient.

Summary of the Invention

A first aspect of the present invention is a method of altering the phenotype of a bird. The method comprises introducing a DNA sequence into somatic cells of a bird contained within an egg during *in ovo* incubation, with the DNA sequence being effective to cause a change in phenotype in said bird after hatch (e.g., a change in growth rate, feed efficiency, disease resistance, or a combination of all of these factors). Introduction of the DNA may be carried out by any suitable means, including injecting the DNA sequence *in ovo* into any compartment of the egg including the body of the embryo. Preferably, the egg into which the DNA is introduced is incubated to hatch, and the bird so produced raised to at least an age at which the change in phenotype is expressed.

In an illustrative embodiment of the foregoing, the DNA sequence is introduced by first transfecting avian hematopoietic progenitor cells with the DNA sequence *in vitro*, and then injecting said transfected hematopoietic progenitor cells into the egg, preferably into the yolk sac or onto the chorioallantoic membrane, and preferably during early embryonic development.

A second aspect of the present invention is a method of altering the phenotype of a bird comprising

A fifth aspect of the present invention is a bird produced by the foregoing methods.

A sixth aspect of the present invention is the use of a DNA sequence for the preparation of a medicament for producing a phenotypic change in a bird by introducing the medicament in ovo, as described above.

A seventh aspect of the present invention is an apparatus for the introduction of a DNA sequence in an egg during in ovo incubation, the DNA sequence capable of producing a phenotypic change in the bird carried by the egg after hatch, as described above.

Detailed Description of the Invention

There are several aspects of avian embryonic development which make it an attractive target for DNA introduction by stem cell transfer. First, since the greatest period of embryonic development occurs in the egg outside the maternal reproductive tract, the embryo can be easily accessed for the introduction of exogenous DNA.

Second, the fact that the egg is a multi-compartmentalized unit can be exploited to deliver biological materials to specific embryonic sites. For example, the yolk sac in the early embryo functions to manufacture blood. Immediately prior to hatching, the yolk sac serves a primarily nutritional function and is taken into the intestinal tract and thereby transported to the cecal pouches during and after hatch. Therefore, yolk sac administration of materials can lead to both embryonic cecal or vascular system delivery. Vascular system delivery through administration of DNA into the yolk sac would be particularly desirable for administering DNA constructs capable of expressing physiologically active peptides in the bird, such as growth hormone, lymphokines such as interferon and interleukin-2, insulin-like growth factor, thyroid releasing hormone (TRH) or epidermal growth factor. In

physiological response (e.g., an immune response, or a hormonal or endocrine response) in a bird after hatch through administering to a bird in ovo a DNA molecule encoding and expressing a protein or peptide, which DNA molecule is administered in an amount effective to induce said physiological response after hatch. Note that the physiological response may be directly induced after hatch, or may be indirectly induced after hatch (such as by induction of a physiological response prior to hatch which endures after hatch), or be a constitutive expression initiated prior to hatch.

A particular altered phenotype of interest is a change in immune response wherein introduction of an avian embryo cell containing a DNA molecule immunizes the bird. Exemplary DNA molecules for introduction are those that encode a protective antigenic protein or peptide that induces an immune response from the recipient bird. This can be done in combination with or in lieu of vaccination of the bird to protect against a specific pathogen.

Altering the endogenous immune response of a bird in ovo is of particular interest due to the presence of maternal antibodies in embryonic and young mammals and birds. Maternal antibodies can interfere with typical vaccination programs for these animals. These antibodies, provided to the neonate from the bloodstream of the mother, conjugate with specific antigens and thus provide natural protection against those antigens prior to the development of immunocompetence by the neonate. Unfortunately, maternal antibodies can also hinder typical vaccination protocols; they bind to the immunogenic component of the vaccine and thus inhibit neonatal production of antibodies. The presence of maternal antibodies precludes vaccination early in the development of the neonate. Typically, multiple vaccination protocols are required so that active immunization can occur once maternal antibody levels have

the field after hatch. The timing and the duration of the last quarter of incubation varies among different avian species due to the variation in incubation duration. For example, for chickens, the last quarter of incubation is from about day 16 to hatch; for turkeys, the last quarter is from about day 19 to hatch.

Other altered phenotypes of particular interest include modification of size, growth rate, feed efficiency, metabolic rate, endocrine response, neural system structure and function, and gender.

B. Gene Targeting

1. Stem Cell Injection

As used herein, the term "embryonic stem cell" is intended to refer to embryonic cells that are uncommitted to any differentiation path, or "totipotent"; i.e., their ultimate function in the mature bird is undetermined, as they can differentiate along any cell lineage pathway and terminally differentiate into any mature cell type. It is generally believed that embryonic stem cells exist in the embryo up to the developmental stage at which the embryo, still a blastula, comprises between about 8 and 64 cells. The term "tissue-specific stem cell" refers to an embryonic cell which is developmentally committed to a particular tissue type, but which can still differentiate into one of a plurality of cell types within the tissue (i.e., are "pluripotent"), and which retain the ability to self-renew. Exemplary tissue-specific stem cells are primoridal germ cells and somatic stem cells; somatic stem cells include, but are not limited to, hematopoietic stem cells, which differentiate to form the mature cells of the lymphocytic and myelocytic lineages, and neural crest stem cells, which differentiate to form portions of the nervous system and melanocytes. The term "non-tissue-specific stem cells" refers to self-renewing cells which are no longer totipotent, but which nonetheless are

Embryonic stem cells have been used effectively as cellular vehicles in mice as a means to produce any desired genotype (Capecchi, TIG, 5, 70-76 (1989)). A major advantage of using cells as vehicles for gene transfer is that the incorporation and function of the gene can be evaluated in vitro without screening vast numbers of animals. In addition, gene transfer in birds is most likely to be of value to the poultry industry if the modifications of the genome occur in a specific, site directed manner rather than by a random approach. Targeting of introduced genes to specific sites in the chromosome can be achieved using gene constructs that are capable of undergoing homologous recombination, in which exogenous and native DNA molecules recombine within regions of homology. The attraction of this approach lies in its potential for modification of endogenous genes in situ to enhance or eliminate expression, to alter tissue specificity, or to alter developmentally regulated expression.

Another donor cell type that can be effectively transferred is the primordial germ cell. Such cells can be isolated from the embryonic blood of Stage XVI embryos (55-60 hours of incubation). This is because these cells originate outside the embryo in the germinal crescent and migrate via the blood to the germinal ridge, which is the future site of the gonad. Simkiss et al., Protoplasma 151, 164-166 (1989) have demonstrated that primordial germ cells containing endogenous retroviral molecules can be transferred to comparable recipient Stage XVI embryos that lack this marker. The introduction of foreign DNA through primordial germ cells can lead to alteration of a number of phenotypic expressions, including gender.

Further, phenotype can be altered by the introduction of somatic tissue-specific stem cells. In particular, hematopoietic stem cells introduced in ovo can colonize the bone marrow and thereafter migrate into the peripheral blood of the embryo. Such colonization

introducing the cells at the stage in the developmental cycle in which such cells are colonizing target tissue.

An established avian muscle cell line has been shown suitable for introducing cloned transgenes into recipient embryonic muscle cells (Antin et al., Devel. Biol 143, 122-129 (1991); Antin and Ordahl, Devel. Biol 143, 111-121 (1991)).

2. DNA Constructs

The DNA molecule introduced in ovo is, in general, a construct contained within an embryo cell comprising a promoter functional in avian cells and a gene encoding a peptide or protein operably linked to the promoter. Preferably, the protein or peptide is physiologically active and capable of producing a phenotypic change in the bird. In general, the DNA construct may be a linear DNA molecule or a molecule carried by a vector or other suitable carrier such as liposomes, calcium phosphate, or DMSO. Vectors, as discussed below, may be plasmids, viruses (including retroviruses), and phage, whether in native form or derivatives thereof. Preferably, the DNA molecule does not contain retroviral DNA portions sufficient for integration of the infecting DNA into the chromosomal DNA of the host bird.

Illustrative of genes encoding a protein or peptide are those which encode a protein or peptide selected from the group consisting of growth hormone, thyroid releasing hormone (TRH), lymphokines such as interferon and interleukin-2, insulin-like growth factor, epidermal growth factor, and immunogenic recombinant antigens such as those produced by Marek's, infectious bronchitis, mycoplasma, avian leucosis, reovirus, pox, adenovirus, cryptosporidia, chicken anemia agent, *Pasteurella* species, avian influenze, Marek's MDX, Gumboro Disease virus, Newcastle Disease Virus (NDV),

each. (Mar and Ordahl, Proc. Natl. Acad. Sci. USA 85, 6404-6408 (1988)). Other exemplary promoters operable in avian cells and embryo cells include promoters associated with the genes expressing skeletal actin, phosphoglycerate kinase (PGK), dihydrofolate reductase (DHFR), and chicken β-globin, promoters for hematopoietic stem cell antigens, promoters operably associated with hematopoietic transcription factors, the promoter for Herpes Virus, thymidine kinase and promoters for viral long-terminus repeats, such as Rous Sarcoma Virus.

Vectors comprise plasmids, viruses (e.g. adenovirus, cytomegalovirus), phage, and DNA fragments integratable into the host genome by recombination. The vector replicates and functions independently of the host genome.

C. Subjects and Time of Administration

The present invention may be carried out on any avian subject, including, but not limited to, chickens, turkeys, ducks, geese, quail, and pheasant. The embryo cells may be introduced in ovo at any time during incubation, the duration of which will vary depending upon the species. For example, DNA may be introduced into chicken eggs early in incubation (e.g., between about days 2 and 3 of incubation) or late in incubation (e.g., during the last quarter of incubation; i.e., between about day 16 of incubation and hatch).

It is preferred that the timing of embryo cell introduction coincide with the embryonic developmental stage in which the introduced cell colonizes in the the embryo. For example, if a hematopoietic stem cell is introduced to a chick embryo, it is preferred that it be introduced between about day 15 and day 17 of incubation, as it is during this stage that the endogenous hematopoietic stem cells of chick embryos typically colonize the bone marrow. By timing introduction thusly, the probability that the foreign cells will colonize also

and position of the embryo, a one-inch needle will terminate either in the fluid above the chick or in the chick itself. Yolk sac injection can be achieved by insertion of a needle to a depth of between about 1/2 and 1 1/2 inches into the [side?] portion of the egg. Air cell injection can be carried out by injection at a depth of between about 1/8 and 1/2 inches into the large end of the egg. Those skilled in this art will appreciate that the injection depth can vary depending on the developmental stage of the embryo. A pilot hole may be punched or drilled through the shell prior to insertion of the needle to prevent damaging or dulling of the needle. If desired, the egg can be sealed with a substantially bacteria-impermeable sealing material such as wax or the like to prevent subsequent entry of undesirable bacteria.

It is envisioned that a high speed automated injection system for avian embryos will be particularly suitable for practicing the present invention. Numerous such devices are available, exemplary being the EMBREX INOVOJECT™ system (described in U.S. Patent No. 4,681,063 to Hebrank), and U.S. Patents Nos. 4,040,388, 4,469,047, and 4,593,646 to Miller. The disclosure of these references and all references cited herein are to be incorporated herein by reference. All such devices, as adapted for practicing the present invention, comprise an injector containing the DNA as described herein, with the injector positioned to inject an egg carried by the apparatus with the DNA. In addition, a sealing apparatus operatively associated with the injection apparatus may be provided for sealing the hole in the egg after injection thereof.

The currently preferred apparatus for practicing the present invention is disclosed in U.S. Patent No. 4,681,063 to Hebrank and U.S. Patent No. 4,903,625 to Hebrank, the disclosure of which are incorporated herein by reference. This device comprises

(1987)). ADH expression is examined in fixed tissues using 2-butanol (Ordahl, *supra* (1986)), a substrate which is specific for Drosophila ADH and cannot be used by vertebrate ADH. Therefore, endogenous expression is able to be distinguished from exogenous expression.

When a construct is expressed, the other injected embryos are allowed to hatch and are raised to 1-2 weeks of age. At various points during this time, the birds are sacrificed and the portion of muscle corresponding to the site of injection and expression in the 19-21 day embryos is analyzed for bacterial β-galactosidase or Drosophila AHD activity.

EXAMPLE 2

Introduction of Hematopoietic Progenitor Cells In Ovo

A DNA-liposome complex consists of 25-100 μg of recombinant DNA as described in Example 1 above and 100 μl Lipofectin™ (Gibco/BRL) formed into liposomes in accordance with known techniques. Aortic hematopoietic progenitor cells are cultured from dissociated aorta cells obtained from embryos at 3 days of incubation in accordance with known techniques. These cells are transfected in vitro with DNA-liposome complexes in accordance with known techniques and injected into the yolk sac or chorio-allantoic membrane of recipient chicken embryos in ovo at 2-3 days of incubation with an Inovoject™ injection apparatus (Embrex, Inc., Morrisville, North Carolina). These embryos are incubated to hatch and the activity of the transgene assessed in bone marrow cultures and blood cells at various intervals post-hatch, utilizing the analytical techniques described in Example 1 above.

delivery sites required that a hole be punched in the eggshell. Delivery to the air cell membrane was accomplished by dripping donor cells in PBS onto the membrane, while delivery to the yolk sac required direct penetration of the yolk sac with a needle. Holes in the eggshell were sealed by plastic wrap anchored by petroleum jelly, and the embryos were allowed to hatch.

Cells were transferred to 82 day 16 embryos: 41 embryos received donor cells on the air cell membrane, and 41 embryos received donor cells in the yolk sac. Twenty-eight of the 41 embryos receiving cells in the yolk sac hatched (68% hatch percentage), and 37 of 41 embryos receiving donor cells on the air cell membrane hatched (90% hatch percentage).

One week after injection, blood samples were obtained by puncturing the frontal sinus of the bird and collecting peripheral blood in syringes coated with 0.5 M EDTA solution (pH 8.0). Chicks were then euthanized by CO_2 and the sex of each was determined visually. Blood samples from males were then used in the PCR and DNA hybridization studies that follow.

EXAMPLE 4

PCR Analysis to Detect the Presence of DNA from Donor Females in the Blood of Recipient Males

A 1 μL sample of recipient blood collected by the procedure of Example 3 was used in the PCR with primers specific for the W-chromosome specific repetitive sequence. The PCR analysis was carried out according to standard techniques, with a positive PCR signal indicating that female donor cells were present in male recipients. A total of 45 male chicks were analyzed by PCR. The data are shown in Table 1.

minutes at room temperature followed by two 15 minute washes in 5 mM $MgCl_2$ in PBS(-) at room temperature. Samples were dehydrated by washing in 70% and 95% ethanol for 5 minutes each at room temperature, then were air-dried. DNA on the slides was denatured by incubation in 70% (v/v) formamide, 2xSSC pH 7.0 at 73°C for 8 to 10 minutes. Slides were then immediately washed in 70% ethanol at -20°C for 5 minutes, washed again for five minutes in 95% ethanol at -20°C, and air dried.

A hybridization solution was prepared containing 50% (v/v) formamide, 2xSSC pH 7.0, 10% (w/v) dextran sulfate, 1% (v/v) Tween-20, 100 ng/μL denatured salmon sperm DNA, and 0.5-1.0 ng/μL labelled W-chromosome-specific DNA probe. The W-specific probe was labelled by incorporation of digoxigenin-substituted nucleotides using a commercial kit (Boehringer Mannheim).

To effect hybridization, the hybridization solution was denatured at 73°C for ten minutes, then both the hybridization solution and the slides were incubated at 37°C for 5 minutes. Twenty μL of hybridization solution was placed on the slide, covered with a siliconized cover slip, and sealed with rubber cement. The slides were then incubated overnight at 37°C in a moist environment. The following day the coverslips were removed by soaking the slides in 50% (v/v) formamide, 2xSSC pH 7.0 at 45°C. Slides were washed three times for 10 minutes each at 45°C in 50% (v/v) formamide, 2xSSC pH 7.0, then washed three more times for ten minutes each in the 2xSSC pH 7.0 alone at room temperature.

Hybridization of the detector probe to the W-chromosome was detected using a colorimetric assay for an alkaline phosphatase-conjugated antibody specific to digoxigenin according to the manufacturer's directions (Boehringer Mannheim). Cells on the slides stained purple indicated the presence of female specific DNA sequences. All experiments included positive controls comprising slides of samples drawn from female chicks.

embryos can persist for up to one week post-transfer, and can be detected in the peripheral blood of post-hatch chicks. In no instance during the procedures of Examples 3-6 were donor cells administered directly into the circulatory systems of embryos. The delivery of hematopoietic stem cells to the air cell *in ovo* is particularly attractive in that an acceptable level of hatchability was maintained; hatchability of about 90% was observed. The ability to deliver a genetically engineered stem cell *in ovo* while maintaining good hatchability makes this a feasible approach to delivery of foreign genes and proteins encoded by those genes to the embryo.

The foregoing examples are illustrative of the present invention, and are not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

8. A method according to claim 1, wherein said somatic tissue-specific stem cells are neural crest cells.

9. A method according to claim 1, wherein said somatic tissue-specific stem cells are coupled with at least one liposome in a DNA-liposome complex.

10. A method according to claim 1, further comprising the step of incubating said egg to hatch.

11. A method according to claim 11, further comprising the step of raising said bird to at least an age at which said change in phenotype is expressed.

12. A method of altering the phenotype of a bird comprising introducing avian embryo cells into the air cell of an egg containing a bird during *in ovo* incubation, said embryo cells containing and capable of expressing at least one DNA molecule in an amount effective to cause a change in the phenotype of the bird.

13. A method according to claim 12, further comprising the step of transfecting said embryo cells with said DNA sequence prior to said introducing step.

14. A method according to claim 12 wherein said bird is selected from the group consisting of chickens, turkeys, ducks, geese, quail and pheasants.

15. A method according to claim 12 wherein said avian cells are introduced *in ovo* during about the last quarter of incubation.

16. A method according to claim 12, wherein said embryo cells are introduced *in ovo* at a stage of

25. A method according to claim 22 wherein said somatic tissue-specific stem cells are introduced *in ovo* during about the last quarter of incubation.

26. A method according to claim 22, wherein said change in phenotype comprises an increase in growth rate, feed efficiency, disease resistance.

27. A method according to claim 22, wherein said embryo cells are introduced *in ovo* at a stage of development in which said stem cells colonize tissue of said bird.

28. A method according to claim 22, wherein said somatic tissue-specific stem cells are hematopoietic stem cells.

29. A method according to claim 22, wherein said somatic tissue-specific stem cells are neural crest stem cells.

30. A method according to claim 22, wherein said somatic tissue-specific stem cells are coupled with at least one liposome in a DNA-liposome complex.

31. A method according to claim 22, further comprising the step of incubating said egg to hatch.

32. A method according to claim 32, further comprising the step of raising said bird to at least an age at which said change in phenotype is expressed.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/00762

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 5/00, 5/06, 5/16, 15/00, 15,06
US CL :435/69.1, 172.3, 240.1, 320.1; 800/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 172.3, 240.1, 320.1; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 90/03439, (Bosselman, R.A. et al.) 05 April 1990, see entire document, especially pages 2-6.	1-32
Y	Protoplasma, Volume 151, issued 1989, Simkiss, K. et al., "Transfer of Primordial Germ Cell DNA between embryos", pages 164-166, see entire document, especially page 164-165, Materials and methods.	1-32

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

01 April 1993

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INTERNATIONAL SEARCH REPORT

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B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN-Medline, Dialog-Medline
Search Terms: Transgen?, Stem Cell?, hematopoietic?, neural crest, PGC, primordial germ cell?, avian?, bird?, chicken?, turkey?, goose, geese, quail?, pheasant?, transplant?, chimera? chimaera?